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GENETIC AND SEROLOGICAL CHARACTERISTICS OF TISSUE-SPECIFIC AUTOIMMUNE DISEASE

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Genetic and serological characteristics of tissue-specific autoimmune disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

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ABSTRACT

The immune system has evolved to protect us from infectious disease and not to overreact to our own tissues or commensal flora. Immune system attack directed against self-tissue is referred to as autoimmune disease, a group of diseases that affect more than 5% of the population. Hypothyroidism and type 1 diabetes are well-known examples, resulting from the destruction of the thyroid gland, and of the insulin-producing beta cells in the pancreatic islets, respectively. Autoimmunity is also the predominant cause of primary adrenal failure, known as Addison's disease, where the adrenal cortex is destroyed by the immune system. All four studies in this thesis were aimed to improve our understanding of autoimmune disease in terms of genetic risk factors, serological biomarkers, and tolerance mechanisms. Despite its high heritability, little is known about the genetic background of Addison's disease. Paper I and II address the heritable risk factors in Addison's disease and discover novel genetic risk variants. Early recognition of the rare syndrome autoimmune polyendocrine syndrome type I (APS1) is essential for prevention of its potentially lethal complications. By identifying four previously undiagnosed patients with APS1, Paper III is a proof-of-concept study showing that serological screening of patients with Addison's disease can identify otherwise undiagnosed APS1 patients. Paper IV studies peripheral immune tolerance by investigating the serologic repertoire in patients with mutations in *FOXP3*, lacking regulatory T lymphocytes. Autoantibodies against a set of structurally unrelated enterocyte antigens are demonstrated, including tissue-specific nuclear receptors. In summary, this thesis takes on several aspects of autoimmunity. Both genetic, serological and clinical studies are integrated to explore new characteristics of tissue-specific autoimmune disease.

LIST OF SCIENTIFIC PAPERS

I. Eriksson D*, Bianchi M*, Landegren N, Nordin J, Dalin F, Mathioudaki A, Nordling Eriksson G, Hultin-Rosenberg L, Dahlqvist J, Zetterqvist H, Karlsson Å, Hallgren Å, Farias F. H. G, Murén E, Ahlgren K. M, Lobell A, Andersson G, Tandre K, Dahlqvist S. R, Söderkvist P, Rönnblom L, Hulting A.-L, Wahlberg J, Ekwall O, Dahlqvist P, Meadows J. R. S, Bensing S, Lindblad-Toh K, Kämpe O†, Rosengren Pielberg G.†
*† Equal contributions

Extended exome sequencing identifies BACH2 as a novel major risk locus for Addison's disease

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II. Eriksson D, Bianchi M, Landegren N, Dalin F, Skov J, Hultin-Rosenberg L, Mathioudaki A, Nordin J, Hallgren Å, Andersson G, Tandre K, Dahlqvist S, Söderkvist P, Rönnblom L, Hulting A-L, Wahlberg J, Dahlqvist P, Ekwall O, Meadows J, Lindblad-Toh K, Bensing S, Rosengren Pielberg G, Kämpe O

Common genetic variation in the autoimmune regulator (AIRE) locus is associated with autoimmune Addison's disease

Manuscript in revision.

III. Eriksson D, Dalin F, Nordling Eriksson G, Landegren N, Bianchi M, Hallgren Å, Dahlqvist P, Wahlberg J, Ekwall O, Winqvist O, Catrina S-B, Rönnelid J, The Swedish Addison Registry Study Group, Hulting A-L, Lindblad-Toh K, Alimohammadi M, Husebye E. S, Knappskog P. M, Rosengren Pielberg G, Bensing S, Kämpe O

Cytokine Autoantibody Screening in the Swedish Addison Registry Identifies Patients With Undiagnosed APS1

Journal of Clinical Endocrinology and Metabolism, 2018, **103**(1): 179-186.

IV. Eriksson D, Bacchetta R, Gunnarsson H. I, Chan A, Barzaghi F, Ehl S, Hallgren Å, van Gool F, Sardh F, Laakso S. M, Rönnblom A, Ekwall O, Mäkitie O, Bensing S, Husebye E. S, Anderson M, Kämpe O, Landegren N

Patients deficient in regulatory T cells target enterocyte antigens

Manuscript

CONTENTS

ABBREVIATIONS

AA	Alopecia areata
AAD	Autoimmune Addison's disease
ACTH	Adrenocorticotropic hormone
AIHA	Autoimmune haemolytic anaemia
AIRE	Autoimmune regulator
AITD	Autoimmune thyroid disease
APS1	Autoimmune polyendocrine syndrome type 1
AS	Ankylosing spondylitis
BACH2	BTB Domain And CNC Homolog 2
CD	Crohn's disease
CoD	Coeliac disease
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CYP27B1	Cytochrome P450 Family 27 Subfamily B Member 1
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
eQTL	Expression quantitative trait locus
FAS	Fas cell surface death receptor
FOXP3	Forkhead box P3
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
IgG	Immunoglobulin G
IL2RA	Interleukin 2 receptor subunit alpha
IPEX	Immunodysregulation, polyendocrinopathy, and enteropathy, X-linked
ITCH	Itchy E3 ubiquitin protein ligase
JIA	Juvenile idiopathic arthritis
LRBA	LPS responsive beige-like anchor protein
MG	Myasthenia gravis
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NLRP1	NLR family pyrin domain containing 1
NOD2	Nucleotide binding oligomerization domain containing 2
PBC	Primary biliary cirrhosis
PCR	Polymerase chain reaction
PSO	Psoriasis
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
QTL	Quantitative trait locus

RA	Rheumatoid arthritis
RAG	Recombination-activating genes
RNA	Ribonucleic acid
SLE	Systemic lupus erythematosus
SNP	Single-nucleotide polymorphism
SS	Sjögren's syndrome
STAT	Signal transducer and activator of transcription
T1DM	Type 1 diabetes mellitus
TSH	Thyroid-stimulating hormone
UC	Ulcerative colitis
UTR	Untranslated region
VIT	Vitiligo

INTRODUCTION

AUTOIMMUNE DISEASE

The essential characteristic of autoimmunity is the immunological attack on self-tissue [1]. What triggers the immune system to mount a specific response against self-antigens is largely unknown, but the importance of both environmental and genetic factors, especially HLA genotype, have been demonstrated. The incidence of different autoimmune diseases varies throughout the world, but collectively affect about 5% of the population [2-4]. It is a diverse group of clinically distinct entities, and the amount of evidence supporting an autoimmune aetiology varies between different diseases [5].

To investigate whether a disease is autoimmune is easier said than done. A number of criteria can be evaluated, but perhaps the most obvious autoimmune feature is pathogenic autoantibodies. Transfer of disease by passive immunization gives direct evidence of autoimmunity [6]. For obvious reasons, transfer of pathogenic autoantibodies between humans is avoided but it sometimes occurs as an experiment of nature. Transplacental transmission of IgG occurs in every pregnancy and can transfer autoantibody-mediated diseases from mother to foetus. That is the case with thyrotropin receptor antibodies in neonatal Graves' disease and acetylcholine receptor antibodies in neonatal myasthenia gravis [7]. Transfer of autoantibodies across species can also demonstrate their pathogenic effect [8]. Autoantibodies in most autoimmune diseases, however, are not pathogenic in themselves, and the tissue damage is believed to be inflicted by antigen-specific T cells. In line with this, Addison's disease and type 1 diabetes mellitus are not transferred from afflicted mothers to their foetuses, despite placental transmission of autoantibodies against 21-hydroxylase and GAD-65, respectively.

Even in the absence of pathogenic autoantibodies, it is possible to find indirect evidence of an autoimmune aetiology. An early model dates back to the nineteen-fifties and resemble Koch's postulates for infectious disease [9]. Like a microbial agent can be identified and employed to transfer disease between individuals, Witebsky suggested analogous criteria to support an autoimmune aetiology: the B cell autoantigen should be identified, and immunization with the same antigen in experimental animals should reproduce both the disease and the autoantibodies. The mere demonstration autoantibodies or self-reactive T cells targeting the affected organs, also provide indirect evidence of autoimmunity [10, 11]. The suspicion of autoimmune disease aetiology can also be raised by circumstantial evidence such as beneficial response to immunosuppressive therapy or a strong association to HLA genotype [5]. Infiltration of immune cells in affected tissues are also common findings, although not specific for

autoimmunity [12]. Concomitance with other autoimmune diseases in the same patient or the same family is typical.

An autoimmune disease can be classified according to its distribution as either *systemic*, exemplified by SLE, or *organ-specific* in for instance autoimmune Addison's disease and type 1 diabetes mellitus [13]. Systemic diseases can for long be limited to a single tissue but they can also flare with multiorgan involvement and fever. Autoantibodies in organ-specific autoimmune diseases are typically directed against tissue-restricted proteins, whereas in systemic diseases, they target ubiquitously expressed proteins. The group of organ-specific autoimmune diseases can be further subdivided (Table 1). In *destructive* autoimmune diseases such as type 1 diabetes and Addison's disease the islet cells and adrenal cortex, respectively, are permanently destroyed and the essential hormones they produce are lost. In contrast, the *non-destructive* autoimmune diseases can disrupt the function of cell surface proteins without destroying the target tissue. In Graves' disease, autoantibodies *stimulate* the TSH-receptor resulting in thyrotoxicosis, while in myasthenia gravis, autoantibodies *inhibit* the acetylcholine receptor in the neuromuscular junction resulting in muscle weakness.

Table 1. Characteristics of organ-specific autoimmune diseases.

	Example of diseases	B cell autoantigen
Destructive autoimmunity	Addison's disease	21-hydoxylase
	Type 1 diabetes	Glutamate decarboxylase-65
	Autoimmune thyroiditis	Thyroid peroxidase
Non-destructive autoimmunity	Graves' disease	TSH receptor
	Myasthenia gravis	Acetylcholine receptor
	Limbic NMDA receptor encephalitis	NMDA receptor

THE GENETIC BACKGROUND OF AUTOIMMUNITY

Autoimmune diseases sometimes co-occur in families, even across diseases, and the increased incidence is far higher than expected by chance [14]. For example, ulcerative colitis often co-occurs with Crohn's disease [15], and vitiligo with both coeliac disease and type 1 diabetes [15]. More than half of patients with Addison's disease have a concomitant autoimmune disease with autoimmune thyroid disease being most frequent followed by autoimmune atrophic gastritis with B_{12} deficiency, type 1 diabetes, premature ovarian insufficiency in women, and vitiligo [16, 17]. The aggregation in families and individuals suggests that risk factors are shared between family members, and between diseases [3]. Even though not much is known in detail, autoimmune disease is described as the result of interaction of both genetic susceptibility factors and

environmental triggers [18]. Environmental risk factors include for instance iodine intake associated with an increased incidence of autoimmune thyroiditis and certain influenza vaccinations associated with narcolepsy [19, 20]. Gene-environment interactions have also been demonstrated, for example smoking with HLA haplotypes and *PTPN22* in multiple sclerosis and rheumatoid arthritis [21-23].

In addition to shared environment, shared genetic risk factors lay the foundation for co-occurrence of diseases. However, although common autoimmune diseases co-occur within families and share genetic risk factors, they show no signs of Mendelian inheritance [2]. The genetic variants associated to disease are found in both affected and unaffected individuals. This ambigous link between genotype and phenotype is typical of complex traits and has several causes. For instance, genes involved in the same biochemical pathway may be distributed throughout the genome, and a mutation in a single one of them may increase disease susceptibility in individual patients. Other diseases may require the joint effects of a number of variants in several genes to manifest; no single mutation is enough to cause the disease and no single mutation is strictly required.

DISSECTING INHERITANCE IN COMPLEX TRAITS

Linking disease traits to genomic markers begun more than a hundred years ago [24], and the recognition of naturally occurring DNA variation was a breakthrough in the number of available markers [25]. Loci conferring the highest disease-risks are the easiest to discover and the HLA complex was consistently linked to autoimmune disease well before the advent of high throughput genotyping, using linkage analysis in family-based studies [26, 27]. Genomic linkage studies take advantage of the fact that alleles on the same chromosome are more likely to be inherited together if they are located close to each other [28]. Throughout the genome, selected markers are genotyped in family members, with and without the trait of interest. By studying the extent that alleles co-segregate with the disease in a pedigree, linkage between a genomic region and disease is demonstrated. Typically, the resultant regions are large since the resolution depends on the number of recombination events, and the size of the studied families. Linkage analysis is most suitable for simple Mendelian traits but has also successfully linked some regions to complex traits, such as *NOD2* to Crohn's disease [29, 30].

In contrast to linkage analysis, association studies do not rely on pedigrees and are often preferred for studies of complex traits. They simply compare the frequencies of an allele or a genotype in affected patients and healthy controls [31]. Hence, a simple contingency table is all it takes to summarize the data for the two study groups [32]. Alleles or genotypes occurring significantly more frequently in patients are associated with disease. Rather than cosegregation in families, association studies depend on unrelated individuals to provide unbiased allele frequency estimates. Carefully chosen case and control groups are essential in order to avoid the otherwise reliable

disappointment of spurious associations [31]. Studies of single candidate-genes or small case groups are prone to false associations caused by both population substructures and chance, and results have often been difficult to reproduce in larger well-powered studies [33]. Nonetheless, the candidate-gene approach has yielded important findings, such as the associations of *CTLA4* and *PTPN22* to several autoimmune diseases including autoimmune Addison's disease (Table 2) [34-38]. With high-throughput genotyping, however, investigators can include markers from the entire genome and are no longer limited to single candidate genes. Studies covering large proportions of the genome are less biased in this sense and enable novel discoveries.

Table 2. Example of risk loci that have been associated to several common autoimmune diseases, and their function.

Gene / Region	Function
HLA-DRB1	Expressed on specialized antigen-presenting cells and present peptides from extracellular proteins to T cells.
CTLA4	Costimulatory molecule transmitting signals from antigen-presenting cells. Inhibits antigen-activated immune responses in T cells.
IL2RA	Receptor for interleukin 2, essential in T cell proliferation and differentiation.
PTPN22	Lymphoid-specific protein tyrosine phosphatase involved in T cell receptor signalling.
STAT4	Intracellular signalling molecule mediating responses to interleukin 12 in lymphocytes, controlling the differentiation of T helper cells.

Immunobase (immunbase.org) and RefSeq (ncbi.nlm.nih.gov/refseq/rsg/) accessed April 11 2018.

Under the null hypothesis, *P* values follow a uniform distribution. With multiple genetic markers studied in parallel, the risk of detecting associations due to chance increases, and the statistical significance threshold has to be adjusted accordingly (figure 1, table 3) [39]. Such a correction can compensate for the number of hypothesis tests at the expense of decreased statistical power. To maintain a sensitivity for risk loci with moderate or small effects, larger sample sizes are required. However, the simultaneous study of many independent markers is advantageous in other aspects. For instance, population substructures can be taken into account when calculating the strength of association. Relatedness between subjects can be thought of as their pairwise mathematical distance measured across all the dimensions that the genetic markers constitute. With dimensionality reduction, the distances between all possible pairs of samples can be summarized to coordinates, easy to plot in two dimensions [40]. Typically, these plots may resemble a geographical map corresponding to the ancestry of the samples, and population substructures are visualised as clusters [41]. By including coordinates as covariates in statistical models, population substructure can be accounted for in multi-marker genetic studies and reduce the risk of false associations.

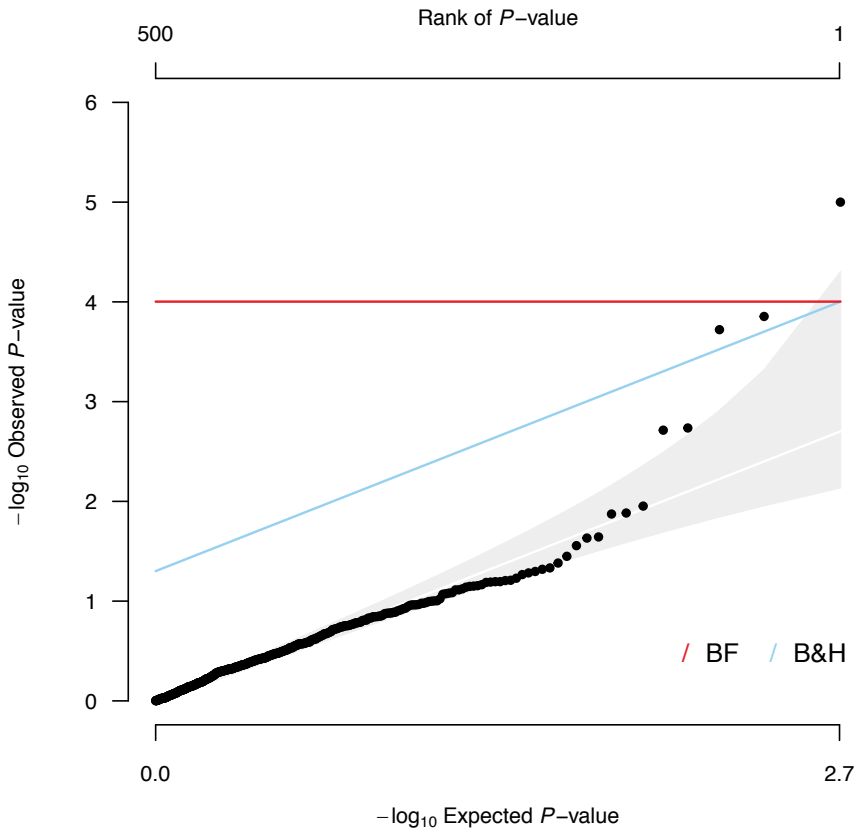

Figure 1. Quantile-quantile plot of 500 *P* values following a uniform distribution. Critical values are depicted according to Bonferroni (BF, red), and Benjamini and Hochberg (B&H, blue). The 95% confidence interval is shaded in grey with a white identity line.

Table 3. Correction for testing m hypotheses at the accepted risk α of making a type I error, conventionally set at 0.05. The false discovery rate depends on the rank (i) of the *P*-value.

Error criterion	Method	Critical value (c)
Familywise error rate	Bonferroni	$c = \alpha / m$
False discovery rate	Benjamini and Hochberg	$c_i = i\,\alpha / m$

Genome-wide association studies (GWAS) typically investigate hundreds of thousands of SNPs across the genome, and are optimized to discover common alleles that explain a significant proportion of the population disease risk [42]. Hundreds of common variants have been associated with autoimmune diseases using GWAS [43]. Some associations are disease-specific, for instance *Thyroid stimulating hormone receptor* in Graves' disease [44] and *Insulin* in type 1 diabetes [3]. Other associations are shared between autoimmune diseases and suggest a common genetic aetiology, in line with the familial clustering across individuals and diseases [3, 45-47]. In fact more than half of the SNPs associated to autoimmune diseases in genome-wide studies associate to more than one of the diseases [47]. Given this large extent with which genetic risk factors are shared between diseases, it is worth noting that common genetics predict co-occurrence rather than clinical similarity. For instance, ankylosing spondylitis and rheumatoid arthritis both affect joints, but show exceptionally few shared risk loci for a pair of autoimmune

diseases [3]. The most consistently associated locus across autoimmune diseases is the HLA region, typically representing the strongest genetic risk factor [48]. Depending on disease, both homozygosity and heterozygosity can confer the highest risks, and different HLA genes and HLA alleles show association with different diseases [49, 50].

WHAT HERITABILITY IS AND IS NOT

It is easier to successfully link a genomic region to disease if the disease at hand has a high heritability [31]. This popular statistic is defined as the variance in the genotype divided by the total variance in the phenotypic trait, and hence range from 0 to 1 (Table 4). For a dichotomous trait such as destructive autoimmunity, this means that for a given population and a given environment, the heritability is the proportion of disease liability that can be attributed to genetic variation. It does, however, *not* tell us the proportion of a trait that will be inherited by the offspring, and a high heritability does *not* allow us to determine the phenotype from the genotype [51].

Table 4. Definition of the heritability estimate.

$$h^2 = \frac{\sigma_A^2}{\sigma_P^2} = \frac{\sigma_A^2}{\sigma_G^2 + \sigma_E^2} \quad (1)$$

Narrow sense heritability (h^2) is defined as a ratio of the additive partition of genotypic variance (σ_A^2), and the variance of the observable phenotypes (σ_P^2) [51].

The variance of observable phenotypes can be partitioned into underlying variances of genotypes and environment (σ_E^2).

With the population prevalence taken into account, the heritability is usually estimated from either the disease recurrence rate in close relatives (λ_R), or from disease concordance rates in identical and fraternal twins [31, 52]. A higher relative risk in closer relatives corresponds to a stronger heritability. For instance, in a twin concordance study by Skov et al. the heritability in Addison's disease in Sweden has been estimated to be 0.97 (95% CI 0.88–0.99) [53]. In this study, the variance in environmental effects have had little influence on total phenotypic variance and the ensuing high heritability indicates a trait suitable for genetic study [31]. Autoimmune diseases coinherited with Addison's disease have shown comparable results in other populations; type 1 diabetes 0.69-0.88 and autoimmune thyroid disease 0.79 [54-59]. Even if, strictly speaking, the heritability is population-specific, it is often similar in different populations [51]. The heritability reveals the relative importance of genes and environment on the variation of disease liability. It can also be used to establish the efficiency of gene-mapping studies.

EFFICACY OF GENETIC ASSOCIATION STUDIES

Autoimmune diseases have been studied in GWAS and the discovered risk alleles typically have a small or modest effect on disease liability, evident from an odds ratio

near 1. For instance, in autoimmune thyroid disease, SNPs outside the HLA region have odds ratios in the lower range; 1.16 – 1.63 [60]. In type 1 diabetes, the range is similar; 1.05 – 2.38 [61, 62](Odds ratios from *immunobase.org,* accessed 16 March 2018). Collectively, GWAS variants explain only a minor fraction of the total heritability, and this is a recurrent finding for several complex traits [42, 63, 64]. The enrichment of genes expressed in certain cell types or essential in certain pathways, may nevertheless render biological hypotheses [65]. Risk loci in type 1 diabetes, for instance, are enriched for genes influencing the adaptive immune response and T cell differentiation [66]. The proportion of heritability that is accounted for by the established risk genes in Addison's disease has not been determined.

The power to detect associations with tiny effect sizes steadily increases with the inclusion of more study subjects. Even in GWAS comprising hundreds of thousands of samples, however, the tiny effect sizes discovered collectively explain less than half of the phenotypic variance [67, 68] . As the study sizes increase, the possible effect sizes in associations left to discover decrease, and the total number of loci necessary to associate in order to explain the full heritability increases towards the hundreds or even thousands. Some researchers nevertheless believe that the majority of remaining risk variants reside in loci with tiny effects, hiding in the background of random associations [69]. Others advocate that rare variants with large effect sizes are the most probable cause of the apparently missing heritability [69]. Whatever the case, undiscovered genetic variants may still exist in complex and repetitive regions of our genome. Copy number variations are often neglected in genetic studies but have been associated with autoimmune diseases such as psoriasis and Crohn's disease [70, 71]. Rare structural variants can collectively make up a substantial contribution to genetic variation [72].

MAKING THE LEAP FROM ASSOCIATION TO CAUSATION

More than 90% of the variants associated to autoimmune diseases in GWAS reside outside of protein-coding genes [46]. They preferentially enrich in regulatory regions active in immune cells but rarely affect recognizable transcription factor binding motifs [46, 73]. For example, risk variants in type 1 diabetes enrich in enhancers active in thymus and lymphocytes. whereas SLE loci control genes preferentially expressed in B cells [62, 73, 74]. Only a fragment of the hundreds of risk loci discovered in GWAS have had their exact molecular mechanisms resolved. Association studies alone have a limited ability to distinguish causal variants from the region associated to disease and to discern the individual regulatory element that mediates risk, or the genes it regulates [69].

Since the SNPs associated in a GWAS may affect genes other than the one closest, eQTLs overlapping a GWAS hit can help connect candidate causal variants with appropriate risk genes [75]. Epigenetic data can also help assess both which variants are likely to be causal [76, 77] , and to identify the regulatory regions that mediate risk and the genes

they control [78]. Integration of genetic associations with expression patterns and epigenetic data may aid in the identification of candidate variants for functional studies [75, 79-81].

HIGH THROUGHPUT SEQUENCING

The sequencing of the human genome was facilitated by the advent of massive parallel sequencing technology and finished in year 2001 [82, 83]. High throughput sequencing is achieved by first shearing the DNA molecules into miniscule pieces, and then sequencing all fragments simultaneously. Each parallel sequencing experiment generates reads, short stretches of genetic code (Table 5). The reference genome helps assemble the reads into contiguous sequences. Mismatches from the reference sequence are listed as genetic variants, and mostly represent natural variation or sequencing errors. Among other things, the final quality of the variant calls is dependent on the read depth, i.e. the number of times each position of the genome has been sequenced and successfully aligned to the reference. Repetitive sequences and structural variation complicate read alignment. To facilitate alignment, DNA fragments are sequenced from both ends, producing pairs of sequencing reads. By aligning paired reads simultaneously, on a fragment length from each other, ambiguous alignments are resolved in complicated regions. Yet, in highly variable regions such as the HLA region, alternative sequencing technologies, or customized methods for aligning and variant calling are advisable.

Table 5. The alignment of sequencing reads to the reference genome enables the recognition of genetic variants. Aligned reads that differ from the reference are the basis for variant calls. In patients that are heterozygous for a given position, about half of the reads are expected to represent each of the two alleles. In this example, the aligned reads indicate a position with a heterozygous genotype. The read depth at this position is 7.

	Unaligned reads	Aligned to reference genome
Sequencing reads	ATGGCATTGCAA TGGCATTGCAATTTG AGATGGTATTG GATGGCATTGCAA GCATTGCAATTTGAC ATGGCATTGCAATTT AGATGGTATTGCAATTTG	ATGGCATTGCAA TGGCATTGCAATTTG AGATGGTATTG GATGGCATTGCAA GCATTGCAATTTGAC ATGGTATTGCAATTT AGATGGTATTGCAATTTG
Reference genome	…AGATGGCATTGCAATTTGAC…	…AGATGGCATTGCAATTTGAC…

Compared to genome-wide SNP-typing, sequencing yields denser variant calls and detects potential rare variants. Sequencing of selected genomic regions reduces costs, compared to a whole-genome sequencing, and can be enabled through targeted enrichment. Exome sequencing is the most common application. Targeted regions are physically captured when complementary probes, attached to solid support, are hybridized to the DNA samples. Hybrid capture can handle thousands of selected sequences but a drawback is the uneven coverage of targeted regions. Highly conserved

exons generally contain fewer variants than the non-coding regions, but more variants with large effect on disease susceptibility [84]. Therefore, exome-sequencing has been the method of choice for identification of rare high-impact variants.

ADDISON'S DISEASE

A typical example of destructive autoimmunity is autoimmune Addison's disease (AAD) [85]. It is the predominating cause of primary adrenal insufficiency and ultimately lethal if the essential adrenal hormones cortisol and aldosterone are not substituted [86, 87]. Many patients present with an acute insufficiency that requires cortisol immediately [88]. Adrenal insufficiency may be suspected in cases with fatigue, low blood pressure, abdominal pain, nausea, weight loss or hyperpigmentation of the skin, and diagnosed in patients with low morning serum cortisol and elevated plasma adrenocorticotropic hormone (ACTH) [87, 89]. In case of uncertainty, inadequate cortisol levels after corticotropin stimulation gives the diagnosis. Autoimmune aetiology is confirmed with the presence of 21-hydroxylase autoantibodies [10, 87]. The onset of AAD is typically occurring in the third to fifth decade of life and slightly more women are affected (60%-65%) [16, 90]. The prevalence in Caucasian populations ranges from 87 to 221 per million. [16, 91-97]. It appears more often in families than what would be expected by chance alone [98-104], in line with a high heritability [53]. The majority of AAD patients have other concomitant tissue-specific autoimmune diseases such as type 1 diabetes, autoimmune thyroid disease, or pernicious anaemia, reflecting shared risk factors [17]. Apart from the well-characterized association to the HLA complex, little has been known about the genetic variants that contribute to disease development [105]. Until now, candidate gene studies have linked genes first implicated in other autoimmune diseases to AAD (Table 6) [105, 106]. The rarity of AAD has, however, made extensive unbiased genome-wide association studies unfeasible.

Table 6. Genes associated to autoimmune Addison's disease in at least two independent candidate-gene studies.

Gene	Study design	Reference
CTLA4	Candidate gene studies	[37, 107-109]
CYP27B1	Candidate gene studies	[110, 111]
NLRP1	Candidate gene studies	[112, 113]
PTPN22	Candidate gene studies	[38, 114-116]

The gap between disease associations and mechanistic knowledge is dramatically shortened when changing focus from diseases with complex inheritance to diseases with monogenic inheritance. Identification of causal genes in rare monogenic autoimmune diseases has enabled the characterization of both normal and pathologic immune system mechanisms.

MONOGENIC AUTOIMMUNE DISEASE

Monogenic autoimmune syndromes have yielded valuable insight into our immune system and its dysregulation in autoimmune disease. For instance, research on autoimmune polyendocrine syndrome type 1 (APS1) has expanded our knowledge about central immune tolerance, enforced by the thymus [117]. Likewise, peripheral immune tolerance has been studied in patients with monogenic defects in regulatory T cells.

Table 7. Rare monogenic diseases have revealed highly penetrant disruptive mutations in critical immune genes. GWA studies point out that common alleles in the same loci have been associated to common autoimmune diseases.

Monogenic autoimmune disease	Affected gene[1]	Complex autoimmune diseases associated in GWAS[2]
Autoimmune polyendocrine syndrome type 1	AIRE	RA
Immune dysregulation due to CTLA4-mutation	CTLA4	AA, AITD, CD, CoD, MG, RA, T1DM, UC
Autoimmune lymphoproliferative syndrome	FAS	JIA
Immunodysregulation polyendocrinopathy, enteropathy, X-linked	FOXP3	-
Immunodeficiency 41 with lymphoproliferation and autoimmunity	IL2RA	AA, AITD, CD, JIA, MS, RA, T1DM, UC, VIT
Multisystem autoimmune disease with facial dysmorphism	ITCH	UC
Common variable immunodeficiency 8 with autoimmunity	LRBA	-
Immunodeficiency 31C	STAT1	CoD, CD, JIA, MS, PBC, RA, SLE, SS, UC
Infancy-onset multisystem autoimmune disease	STAT3	CD, MS, PSO, UC

[1][118-122]. [2]Immunobase (immunbase.org) and GWAS catalog (ebi.ac.uk), accessed March 16 2018. Abbreviations: AA Alopecia areata, AAD Autoimmune Addison's disease, AITD Autoimmune thyroid disease, AS Ankylosing spondylitis, CD Crohn's disease, CoD Coeliac disease, JIA Juvenile idiopathic arthritis, MG Myasthenia gravis, MS Multiple sclerosis, PBC Primary biliary cirrhosis, PSO Psoriasis, RA Rheumatoid arthritis, SLE Systemic lupus erythematosus, SS Sjögren's syndrome, T1DM Type 1 diabetes mellitus, UC Ulcerative colitis, VIT Vitiligo.

Patients with APS1 are classically affected by a triad of clinical manifestations: chronic mucocutaneous candidiasis, hypoparathyroidism and adrenal insufficiency [123, 124]. Common manifestations also include autoimmune forms of diabetes, ovarian failure, alopecia and lung disease [125]. This wide spectrum of disease components is caused by

dysfunction of the AIRE protein, encoded by a single susceptibility gene with the same name [118, 119]. AIRE acts as a transcriptional regulator in thymic cells and it is a key factor in T cell development [117]. With a normal AIRE function, proteins with an otherwise tissue-restricted exoression are ectopically expressed in the thymus. This is instrumental to the negative selection of developing T cells. With a defective AIRE, ectopic expression of proteins in the thymus is disrupted and potential autoreactive T cells evade apoptosis [117, 126, 127]. Although traditionally reported as a recessive disorder, dominant missense mutations have recently been described in a few families, with a less severe phenotype [128-131]. The prevalence of APS1 is roughly 1:100 000 but more common in some countries, primarily due to founder effects and relative isolation [85, 132]. The loss of AIRE function causes autoimmunity in multiple but defined tissues, as evident from both lymphocytic infiltrates and autoantibodies [133, 134].

In contrast to APS1 and *central* tolerance, IPEX (immunodysregulation, polyendocrinopathy, and enteropathy, X-linked) is an autoimmune syndrome that has yielded insight in *peripheral* immune tolerance. IPEX is an X-linked recessive disorder typically presenting in infancy with severe enteropathy [135]. Patients may also acquire additional manifestations including eczematous dermatitis or autoimmune forms of diabetes, thyroid disease, haemolytic anaemia, hepatitis and nephritis [136, 137]. Identification of the causing gene, *FOXP3* [138-140], has been key to understanding the development of regulatory T cells. Regulatory T cells are essential upholders of peripheral self-tolerance, and FOXP3 a transcription factor critical for their differentiation and function [141, 142]. The lack of regulatory T cells in IPEX syndrome causes a loss of peripheral tolerance and autoimmunity in affected patients [120, 143-145]. This makes IPEX a unique model for the study of peripheral immune tolerance.

Several other immune genes have been implicated in monogenic autoimmunity and one can speculate that additional disorders are yet to be discovered (Table 7). Many genes underlying monogenic immune defects are also associated with common autoimmune diseases, even though the pathoetiology in the monogenic disease may differ from their polygenic counterparts [120].

IDENTIFICATION OF NOVEL AUTOANTIGENS

Traditionally, B-cell autoantigens in autoimmune diseases have been identified using western blot of a tissue homogenate or screening of cDNA libraries, both prepared from healthy tissue representing the organ under autoimmune attack. These methods have been utilized in common autoimmune diseases such as Addison's disease [10] and type 1 diabetes [146], as well as in APS1 [147-150] and IPEX [151-154]. However, technical developments have enabled novel explorative approaches for autoantigen discovery. First developed for genetic studies, the technology for printing and scanning DNA microarrays has subsequently been adapted for construction of protein microarrays

[155-157]. With the application of protein arrays, thousands of proteins can be screened in parallel for autoantibodies.

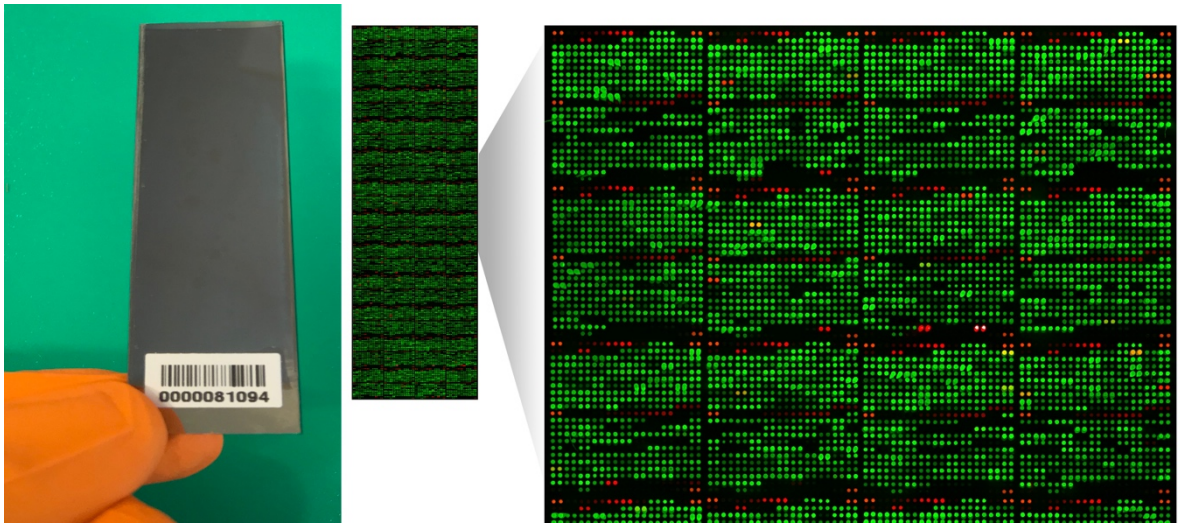

Figure 2. A protein array printed on a microscopy slide. A section of the corresponding scanned image is enlarged and presented to the right.

The first commercially available human proteome array, ProtoArray®, contains more than 9000 full-length human proteins (figure 2). Expressed as GST-fusion proteins using baculovirus vectors and insect cells, the proteins are printed in duplicates on glass slides coated with nitrocellulose. By probing protein arrays with serum samples, potential autoantibodies can interact with their target proteins. Fluorescence labelled anti human-IgG are then used to detect the autoantibodies and anti-GST to visualise the entire set of tagged proteins. The scanned fluorescence images are aligned to a grid that enables data extraction and integration with protein information. By exploring autoantibodies in APS1 sera, that have many well-established autoantigens, Landegren et al. proved the reliability of protein arrays and their unprecedented capacity in discovering novel autoantigens in organ-specific autoimmune disease [133, 158].

AIMS

The aim of this thesis was to characterize tissue-specific autoimmune disease, both in terms of genetic risk factors and serological biomarkers.

Our specific aims were to:

- Identify genetic risk loci in autoimmune Addison's disease (Paper I and II).

- Evaluate the usefulness of cytokine autoantibodies in screening for APS1 patients among patients diagnosed with Addison's disease (Paper III).

- Decipher peripheral immune tolerance mechanisms by investigating autoantibodies in IPEX syndrome (Paper IV).

MATERIALS AND METHODS

Brief overviews of the materials and methods most central to this thesis are described in this section. Detailed descriptions are found in the corresponding papers.

STUDY PARTICIPANTS

The Swedish Addison Registry was started in 2009 to enable comprehensive studies on Addison's disease patients. It is estimated to constitute more than 70% of all putative patients with Addison's disease in Sweden [91]. All included patients have been characterized both clinically and serologically, and about 90% have the autoimmune pathoetiology confirmed with positive 21-hydroxylase autoantibodies [17].

IPEX syndrome is a rare disease and collections of samples are typically small. We have included IPEX patients from collaborators in Germany, the USA, and Italy.

The majority of control subjects were retrieved from blood donors and the remainder from directed sampling of healthy individuals.

NEXT GENERATION SEQUENCING

DNA was extracted from whole blood EDTA and sonicated to fragments of 400 base pairs (bp) for sequencing library preparation. Pools of eight samples were used for hybrid capture and sequenced with 100-bp paired-end reads using Illumina HiSeq 2500. Samples with a mean target coverage of less than 10× were resequenced. Sequencing reads were mapped to hg19 with BWA [159] and processed with a pipeline adapted from genome analysis toolkit (GATK) best practices [160, 161].

PROTEIN ARRAY SCREENING

We used commercial protein microarrays to screen serum samples for autoantibodies (ProtoArray®; Thermo Fisher). Protein arrays were incubated stepwise with serum sample diluted 1:2000, and subsequently with fluorescently labelled detection reagents, according to the manufacturers protocol. Arrays were scanned in a microarray scanner (LuxScan HT24; CapitalBio) and data was extracted using acquisition software (GenePix Pro v6.1; Molecular Devices).

RADIO-LIGAND BINDING ASSAY

To assay serum samples for autoantibodies, we used recombinant proteins marked with a radioactive tracer. Full-length cDNA clones encoding genes of interest were subcloned into expression vectors, transcribed and translated *in vitro*, thereby enabling the incorporation of ^{35}S-methionine. Radiolabelled antigens were incubated with serum

samples, and immune complexes were precipitated with protein-A Sepharose and 96-well filter plates. Radioactive decay was measured in a liquid scintillation counter (Wallac Microbeta 1450; PerkinElmer).

RESULTS AND DISCUSSION

PAPER I

Sporadic autoimmune Addison's disease is caused by an autoimmune destruction of the adrenal cortex, but little is known about the aetiology [162]. Despite the high heritability, no large-scale genetic studies have been undertaken to explore its genetic background. To improve our knowledge about genetic risk factors, we made a comprehensive sequencing study of patients with autoimmune Addison's disease. The Swedish Addison Registry is the world's largest biobank of primary adrenal failure and enabled the inclusion of 700 well-characterized cases. As a control group, 1501 healthy Swedish blood donors were also included.

We developed a capture array for targeted sequencing of a wide range of genes and their regulatory elements. Genes known to be involved in basic immune functions, inflammation, or autoimmune disease, were included as well as additional genes involved in associated immune pathways. Genes causing congenital adrenal insufficiency were also targeted. Besides protein coding exons, we targeted the untranslated regions of gene transcripts, the region surrounding the transcription start site, as well as highly conserved elements in the surrounding 100 kb of the gene boundaries. The conserved elements were identified using genealogical data from 29 mammals [163].

Among other measures taken to avoid false associations, we defined the phenotype thoroughly. We excluded cases where other causes of adrenal failure than sporadic autoimmune Addison's disease could be suspected (n=173), including cases that did not test positive for 21-hydroxylase autoantibodies in two assays, performed at two different laboratories. Cases with autoantibodies against interferon-α and interleukin-22, indicative of monogenic autoimmunity syndromes, were also excluded [164, 165].

Our bioinformatic processing pipeline was set up using the GATK, according to the best practices recommendations. Based on the resulting genotypes, we could estimate ancestry and relatedness of both cases and controls. Predicted non-European samples and first-degree relatives were excluded (n=25) in order to improve case group homogeneity and independence of study subjects. Additional quality control parameters, including overall heterozygosity, X-chromosome heterozygosity and singleton counts, were used to indicate low DNA quality, batch effects and contamination of samples. Taken together, 479 cases and 1394 controls remained for association analyses after quality control.

To associate common single nucleotide variants with disease, we calculated a logistic regression model for each of the 103 120 variants with a minor allele frequency of 1% or more. Coordinates from multidimensional scaling were included as covariates to account for population substructures, and genomic control was applied to compensate for residual inflation of the test statistic [166]. To control for multiple testing, a traditional GWAS significance level was adopted; $P = 5 \times 10^{-8}$. At this level of significance, two associated loci were discovered on chromosome 6 (figure 3). We identified the gene *BACH2* as a novel risk locus in autoimmune Addison's disease (rs62408233-A, OR = 2.01 (1.71–2.37), $P = 1.66 \times 10^{-15}$, MAF 0.46/0.29 in cases/controls), and found two signals of association in the HLA region (rs41315836-G, OR = 0.20 (0.13–0.29), $P = 5.73 \times 10^{-16}$; rs17221059-A, OR = 2.29 (1.84–2.84), $P = 9.59 \times 10^{-13}$. *BACH2* had previously been associated to other organ-specific autoimmune diseases [167-171]. Therefore, we calculated a new logistic regression using only cases with isolated Addison's disease (n=119), and the association in *BACH2* remained statistically significant ($P = 3.9 \times 10^{-8}$). In conclusion, *BACH2* was associated to Addison's disease independently of the many autoimmune comorbidities present in the case group as a whole.

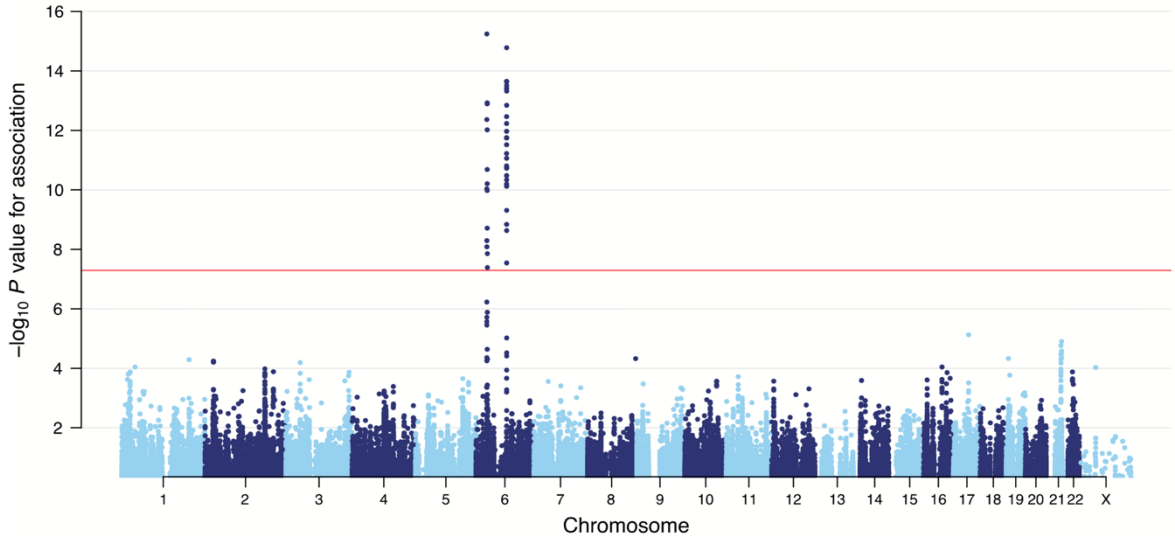

Figure 3. A novel risk locus in Addison's disease was identified on chromosome 6. The two peaks correspond to HLA (left), and *BACH2* (right). Reprinted with permission from Journal of Internal Medicine [172].

The associated SNPs in *BACH2* were all in linkage disequilibrium with each other complicating further dissection of the associated region. They were all located within the untranslated region beginning the gene transcript (5' UTR), which potentially contains regulatory elements. In fact, several of the associated SNPs had already been linked to the expression of *BACH2* in systematic eQTL studies [173]. *BACH2* encodes a transcription factor that operates in lymphocytes. It is an important regulator of antibody class switching and production in B cells [174, 175]. Although not crucial for T cell development in the thymus, it is indispensable for a normal T cell differentiation in

the periphery. With aberrant *BACH2* function, the balance between lineages of helper T cells are disrupted at the expense of regulatory T cells [176, 177]. BACH2 binds to the same DNA motifs as the AP-1 transcription factors, and by antagonizing their activating signals, BACH2 can prevent the terminal differentiation of cytotoxic T cells [176, 178].

BACH2 has been associated to a number of common autoimmune diseases with varying impact, summarized in table 8. Ordered by decreasing odds ratio, *BACH2* has a large effect size in Addison's disease and in coinherited diseases, such as autoimmune thyroid disease and type 1 diabetes. This is perfectly in line with the high co-occurrence observed for these disorders in patients with Addison's disease.

Table 8. Common genetic markers in *BACH2* associated with common autoimmune disesases[1], including our results for Addison's disease.

Disease	Top associated marker	Alleles	Odds ratio
Autoimmune Addison's disease	rs62408233	G>A	2.01
Autoimmune thyroid disease	rs72928038	G>A	1.23
Primary sclerosing cholangitis	rs56258221	A>G	1.23
Type 1 diabetes mellitus	rs72928038	G>A	1.20
Vitiligo	rs3757247	G>A	1.20
Type 1 diabetes mellitus	rs597325	A>G	1.18
Ankylosing spondylitis	rs17765610	A>G	1.15
Multiple sclerosis	rs72928038	G>A	1.14
Coeliac disease	rs10806425	C>A	1.13
Rheumatoid arthritis	rs72928038	G>A	1.13
Coeliac disease	rs7753008	T>C	1.10
Multiple sclerosis	rs12212193	A>G	1.09
Crohn's disease	rs1847472	T>G	1.08

[1]Immunobase (immunbase.org) and GWAS catalog (ebi.ac.uk), accessed March 16, 2018.

To associate rare alleles with complex traits typically requires large sample sets, but can be facilitated by evaluating aggregates of rare alleles collectively [179]. We therefore performed a gene-based association test including variants with minor allele frequencies of 1% or less. However, no additional risk loci were revealed by analysis of rare variants.

Similar to other autoimmune diseases, Addison's disease has been associated to certain HLA haplotypes [180]. Using HLA genotyping from *de novo* assembly of sequencing reads, we could confirm previously known associations to the HLA class II region [181]. The highest risk was associated with the combination of DRB1 alleles 03:01 and 04:04 (OR = 18 (7.2–46), P = 4.9 x 10-12). The confirmation of previously established risk haplotypes underlines the reliability of our sequencing results.

This study used high-throughput sequencing of many of the cases in the Swedish Addison Registry to overcome limitations of studies of single candidate-genes. By also capturing non-coding regions, we were able to associate Addison's disease to

potentially regulatory elements, which are suggested to be major contributors to complex disease [182].Given its association to additional autoimmune diseases and its crucial function in lymphocytes, *BACH2* appears as a major genetic risk factor in organ-specific autoimmunity. Overall, many genes consistently associated to organ-specific autoimmune diseases are essential in lymphocytes. The results of this study are encouraging for future genetic studies in autoimmune disease.

PAPER II

In 2016, allele counts from the whole genome sequencing of 1000 Swedes were made available online. This enabled a good opportunity for a reanalysis of Addison's disease genetics, a field where large genetic studies are almost never done. By means of haplotypes from the international 1000 genomes project, we augmented the dataset from Paper I with additional imputed genotypes and recalculated the associations with additional controls from the 1000 Swedish genomes.

Multiple risk loci have been associated to Addison's disease in candidate-gene studies, some of which have never been confirmed in later studies. With the most comprehensive dataset in Addison's disease genetics, we first revisited previously suggested risk loci and replicated associations to *CTLA4*, *BACH2*, *PTPN22* and *CLEC16A* [37, 107-109, 116, 183-185]. In contrast, we failed to confirm *CYP27B1* [106], *GATA3* [106], and *PD-L1* [186] as risk loci in our Swedish case group. Whether this discrepancy is due to risk factors varying between populations or chance findings in small case-control studies, remains to be investigated.

We next explored the full set of candidate genes and discovered a novel association encompassing four variants in the *AIRE* gene (rs9983695-C, OR = 0.37 (0.27-0.52), P = 2.1 × 10-8, MAF 0.04/0.11 in cases/controls). The four associated SNPs were all located in introns 5 and 8 and in strong linkage disequilibrium with each other. Could the association be due to APS1 patients in the study? At this point, our case group had already been screened for clinical APS1 manifestations, cytokine autoantibodies and protein-altering *AIRE* mutations, but the novel association to *AIRE* still remained. Taken together, common genetic variation in *AIRE* appeared to have a role not only in monogenic, but also in sporadic Addison's disease with complex inheritance. This finding represents an example of genes linked to both a common autoimmune disease with complex inheritance and a rare autoimmune disease with monogenic inheritance (see Table 7 above) [120].

In complex traits, multiple genetic variants influence the risk of developing disease. To investigate the distribution of risk variants in our cases and controls, we counted risk alleles (0, 1 or 2) at six confirmed risk loci (*BACH2*, *CTLA4*, *PTPN22*, *CLEC16A*, *AIRE*, and HLA). As expected, the proportion of cases and controls varied significantly between

risk allele strata. The dataset contained almost no controls with 10-12 risk alleles, and almost no cases with 0-2 risk alleles. Looking in detail on the risk alleles of each subject, they appeared to act independently and with additive effects.

In total, the confirmed six risk loci analysed in this study, accounted for around 7% of variance in liability of autoimmune Addison's disease, so the vast majority of disease heritability still remains unexplained [53]. Future studies encompassing the whole genome, larger case groups, and structural variants are likely to account for a larger proportion of heritability, but in general, even genome-wide studies of thousands of subjects have had limited success in explaining more than half of the heritability [67, 68]. Hopefully, our findings can aid in understanding the genetic background of Addison's disease and inspire further genetic studies.

PAPER III

A small proportion of Addison's disease cases are caused by autoimmune polyglandular syndrome type 1 (APS1) [124]. Although early identification of patients with APS1 is vital given the high risk of potentially lethal complications, APS1 has remained underdiagnosed [187]. We sought to evaluate cytokine autoantibodies and *AIRE* sequencing in screening for APS1 patients in an assorted Addison's disease cohort. In total 677 patients from the Swedish Addison Registry were screened for autoantibodies against interleukin-22 and interferon-α4. Positive patients were further investigated for clinical disease manifestations, additional APS1-specific autoantibodies, and *AIRE* gene abnormalities.

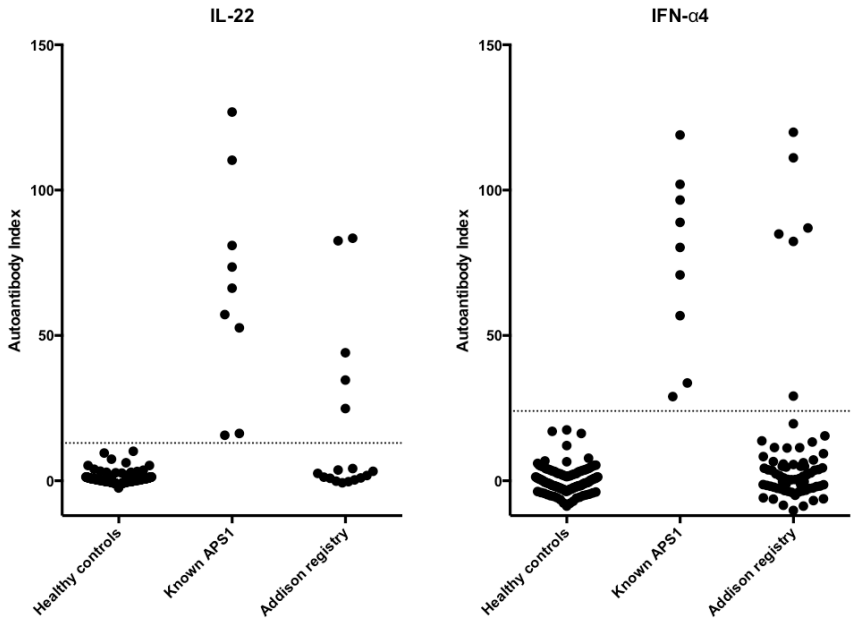

Figure 4. Patients with Addison's disease were screened for autoantibodies against IL-22 and IFN-α4, and compared to healthy controls and patients diagnosed with APS1.

Using radioligand binding assays, we identified 17 patients positive for interleukin-22 and/or interferon-α4, nine of which were already diagnosed with APS1 (figure 4). Sequencing confirmed typical APS1-causing *AIRE* mutations in the nine patients with established APS1 diagnosis. Of the additional eight patients positive for cytokine autoantibodies, four fulfilled clinical criteria for APS1, and hence four new patients with APS1 were identified using autoantibody screening. Investigation of the *AIRE* gene in the four newly diagnosed patients revealed disease-causing mutations in all four patients. One of the patients had none of the previously described missense, splice site or frameshift mutations. In contrast, copy number analysis of the next generation sequencing data revealed a deletion of the first eight exons of *AIRE* (figure 5). Using PCR, the deletion could be confirmed in homozygosity in the same patient, as well as in heterozygosity in three of the other APS1-patients. This finding emphasizes that copy number variations should be kept in mind if sequencing provide no or unexpected results. Serologically, the four newly diagnosed patients had additional autoantibodies against established autoantigens specific for APS1, for instance against SOX10, KCNRG, and AADC [188-190]. This further strengthened the validity of their new diagnoses.

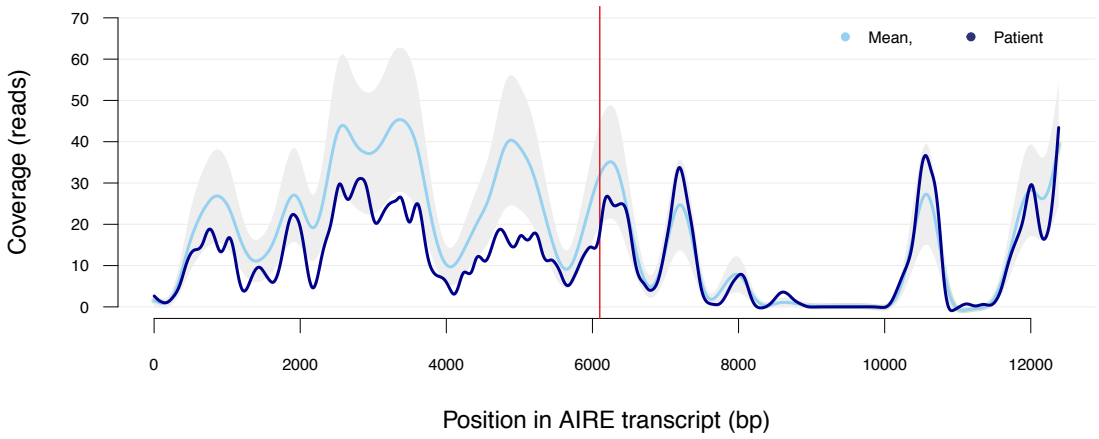

Figure 5. Coverage of *AIRE* is near average for the end of the transcript (right), but about half of average in the beginning of the transcript (left), suggesting a heterozygous deletion.

The clinical APS1 diagnosis requires two of the following three manifestations: Addison's disease, primary hypoparathyroidism, and mucocutaneous candidiasis. However, the genetic and serological aspects have enriched our understanding of APS1 beyond the clinical criteria. Overall, our study showed that additional APS1 patients can be found using serological screening of patients with Addison's disease. Besides the potential importance of the diagnosis for the four newly identified APS1 patients, we hope that future APS1 patients will benefit from the early recognition enabled with autoantibody screening.

PAPER IV

Regulatory T cells depend on the transcription factor FOXP3 for their normal function in peripheral immune tolerance [191, 192]. In IPEX syndrome, caused by FOXP3 mutations, regulatory T cells are dysfunctional and affected patients suffer from multi-organ autoimmunity [143-145]. Type 1 diabetes and severe enteritis are typical manifestations and have been associated with organ-specific autoantibodies [151-153, 193]. We aimed at exploiting the humoral response in IPEX syndrome for identifying novel autoimmune targets. This would reveal what self-proteins rely on peripheral tolerance mediated by FOXP3-dependent regulatory T cells.

We used commercial protein arrays containing over 9000 full-length human proteins to screen for autoantibodies in 14 IPEX patients and 24 healthy controls. Previously established autoantigens Harmonin and GAD65 were both confirmed in the screen. The results were sorted based on case-control differences and the top 20 targets were selected for further investigation. Besides GAD65 that showed independent results, the top 20 targets formed three clusters within which all antigens had highly correlated results: nuclear receptors, enterocyte antigens, and α-interferons. With inclusion of additional IPEX cases and healthy controls, radioligand binding assays allowed us to confirm the reliability and specificity of our novel findings, as well as to test the autoantibodies for cross reactivity.

Nuclear receptors are present in all animals, and the novel autoantibodies were found to be cross reactive against the conserved ligand binding domain [194, 195]. HNF4A was the nuclear receptor with the strongest signals in the array screen. In contrast to other nuclear receptors, HNF4A had a tissue-specific expression profile limited to tissues affected in IPEX syndrome: the gut, kidney and liver. HNF4A is important in the differentiation of intestinal and renal epithelia, and HNF4A knockout mice are characterized by autoimmune enteritis that mimics IPEX enteritis [196-198].

Most IPEX patients suffer from protracted autoimmune enteropathy [199]. It was therefore interesting to note that the second cluster of results contained the established enterocyte antigen Harmonin as well as two additional enterocyte antigens; ANKS4B and ACSL5. ANKS4B interacts with Harmonin to form a structural complex in the microvilli of enterocytes, whereas ACSL5 controls proliferation along the crypt-villus axis [200-203]. Taken together, the autoantigens Harmonin, ANKS4B, ACSL5 and HNF4A all shared the same expression pattern.

As opposed to the tissue-restricted autoantigens previously presented, the α-interferons that constituted the third cluster of results are ubiquitous proteins expressed in response to virus infection [204]. Autoantibodies against α-interferons have been described in patients with APS1, thymoma and patients with RAG-dependent immunodeficiency (Recombination-Activating Genes) [164, 165, 205, 206]. Hence,

interferon autoantibodies are a common feature of immune defects of different etiology. The cause of this and the potential effects remains elusive.

By consecutive sampling of patients undergoing immunosuppressive treatment or hematopoietic bone marrow transplantation (HSCT), we found that autoantibody signals were reduced after immunosuppressive treatment and depleted after transplantation. Repeated sampling may aid the evaluation of IPEX patients undergoing HSCT. We hope that the findings in this study can reflect the normal functions of regulatory T cells and thereby deepen our understanding of peripheral immune tolerance. Despite the fundamental defect in regulatory T cells, the autoantibody repertoire and the clinical manifestations in IPEX syndrome appeared multi-faceted, yet organ-specific.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This thesis investigated different aspects of organ-specific autoimmune disease. The genetic studies presented major novel risk loci in Addison's disease. *BACH2* has been associated to many of the coinherited diseases, whereas *AIRE* has not and is more specific to Addison's disease in this sense. With growing sample collections, we have now reached the time when collaborative efforts could enable genome-wide exploration of common variants in Addison's disease. The identification of previously unknown copy number variations in *AIRE* exemplifies how sequencing can be used to find rare disease-causing genetic variants. Patients with early onset autoimmune disease or cytokine autoantibodies of unknown origin will be interesting candidates for future whole genome sequencing studies.

Autoantibodies typically appear before the onset of clinical manifestations of autoimmune disease. By utilising the high sensitivity and specificity of cytokine autoantibodies, we screened patients with Addison's disease and identified previously undiagnosed APS1 patients. This finding underlines the advantages of measuring autoantibodies, not only when APS1 is suspected, but also for screening purposes. This could prevent potentially lethal complications of APS1 before they occur.

One of the central questions in autoimmunity research, is what governs the immune system's selection of autoantigens. By means of protein arrays, we comprehensively explored the antigen repertoire in patients lacking FOXP3-dependent regulatory T cells. This enabled a comparison of the antigen spectra targeted by the immune system in defects of central (APS1) and peripheral (IPEX) tolerance mechanisms. Characterization of the antigen selection in additional autoimmune diseases, for instance congenital or paraneoplastic autoimmune syndromes, would provide further insight.

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